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Insights into the histology of planarian flatworm *Phagocata gracilis* based on location specific, intact lipid information provided by GCIB-ToF-SIMS imaging

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Abstract

Planarian flatworms are known as the masters of regeneration, re-growing an entire organism from as little as 1/279th part of their body. While the proteomics of these processes has been studied extensively, the planarian lipodome remains relatively unknown. In this study we investigate the lipid profile of planarian tissue sections with imaging Time-of-Flight – Secondary-Ion-Mass-Spectrometry (ToF-SIMS). ToF-SIMS is a label-free technique capable of gathering intact, location specific lipid information on a cellular scale. Lipid identities are confirmed using LC-MS/MS. Our data shows that different organ structures within planarians have unique lipid profiles. The 22-carbon atom poly unsaturated fatty acids (PUFAs) which occur in unusually high amounts in planarians are found to be mainly located in the testes. Additionally, we observe that planarians contain various odd numbered fatty acid species, that are usually found in bacteria, localized in the reproductive and ectodermal structures of the planarian. An abundance of poorly understood ether fatty acids and ether lipids were found in unique areas in planarians as well as a new, yet unidentified class of potential lipids in planarian intestines. Identifying the location of these lipids in the planarian body provides insights into their bodily functions and, in combination

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Author contributions

E.H.C. and L.J.G. planned the project and initial experiment design. T.B.A. and M.J.T. developed sample preparation and analysis plan. T.B.A. performed all microscopy and ToF-SIMS experiments. E.H.C. and N.C. were in charge of animal care and preparation. T.B.A. and D.J.G. analyzed and interpreted the ToF-SIMS data. C.R.A. and C.D.N. were responsible for the LC experiments. T.B.A. prepared all figures and wrote the initial draft of the manuscript. All authors participated in discussion, read, edited, and approved the manuscript.

Transparency document

The **Transparency document** associated with this article can be found, in online version.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbalip.2019.01.011>.

with knowledge about their diet and their genome, enables drawing conclusions about planarian fatty acid processing.

1. Introduction

1.1. Background on planarians

Planarians are non-parasitic, free-living flatworms and members of the class Turbellaria and the phylum Platyhelminthes [1]. Their astonishing ability to regenerate has driven scientists to research the mechanisms behind this capability for > 200 years with early descriptions and studies dating back to the late 18th century [2–4]. This research continues to this day including recent experiments on planarians performed on the International Space Station (ISS) to study the effect of space travel and weightlessness on regeneration [5]. These animals are among the simplest organisms that have three tissue layers, bilateral symmetry and a distinct organ system as well as the earliest form of a central nervous system. In contrast to all other animals possessing these features, planarians have only one body opening, the pharynx, instead of a separate mouth and anus. Famously labelled as being “immortal under the edge of the knife,” [6] these worms have been cut into ever smaller fragments (up to 1/279th of a worm) [7] with each piece regrowing into a perfect copy of the original worm. This is made possible by a pool of pluripotent stem cells (neoblasts) distributed throughout most parts of the planarian body and gradients of proteins expressed to establish the body axes. Neoblasts migrate to the site of tissue damage where the protein environment determines which cell types are needed for repairs. Extensive descriptions on the history of planarian research as well as the current knowledge on their morphology, genetic makeup, protein expression and their mechanism of regeneration can be found in a number of reviews [8–10]. However, little is known about the fatty acid and lipid compositions and distributions in planarians. Although these worms have been investigated for more than two centuries, very little traditional histology data (e.g. hematoxylin and eosin [H&E] stained tissue sections) are available. The knowledge of the morphology of these worms is mainly based on (very detailed) pre-microscopic photography era drawings [11,12], whole body in-situ hybridization staining [13] and fluorescence microscopy studies [14]. Images of H&E stained sections have been primarily from transversal sections and the information they provide depends largely on where the section was taken (central or towards the anterior or posterior end) [15].

1.2. Fatty acids/lipids in planarians

Some reports focusing on lipids/fatty acids in different planarian are available. A study on *Dugesia dorotocephala* by Meyer et al. (1970) suggested that, similar to their parasitic relatives, free living planarian worms rely on dietary fatty acid sources. They can neither de-novo synthesize their own fatty acids nor desaturate dietary fatty acids and can only use acetate for fatty acid chain elongation [16]. In 1992, a study conducted by Politi et al. [17] gave first insights into the lipid profile of planarians (*Dugesia anceps*) by looking at the abundance of different phospholipid species as well as the fatty acids contained in those lipids. Their results indicated that during regeneration processes the overall abundance of any lipid class does not change due to regeneration, however, the lipids in regenerating

worms tend to contain less unsaturated fatty acids. In a similar study, Nery da Matta et al. [18] followed total lipid, triglyceride and cholesterol concentrations up to 192 h after head/tail regeneration in *Dugesia tigrina*. They observed changes in triglyceride concentrations during tail regeneration but did not provide any information on single lipid species. The most recent study was conducted in 2009 by Makhutova et al. [19] looking at fatty acid concentration changes in *Dendrocoelopsis spinosipenis* throughout the year. These authors noted elevated concentrations of fatty acid species FA(22:5) (Docosapentaenoic acid, DPA) and FA(22:6) (Docosahexaenoic acid, DHA) compared to commonly found levels in aquatic organisms as well as the presence of branched/odd-numbered fatty acids usually found only in bacteria. Makhutova et al. speculate that this could be due to a contamination. These odd-chain fatty acids, as well as a high abundance of FA(22:5/6), were also observed in a study by Politi et al. [17]. While possibly coincidental, those observations were made in several different species, collected from a variety of global locations using numerous chromatography methods [17,19,20]. Overall, no study has detailed location specific lipid/fatty acids within planarians and all assumptions about lipid involvement in different planarian bodily functions or regenerative processes to date have been purely speculative.

1.3. Lipid based histology using ToF-SIMS

In this study we have filled the above stated knowledge gaps by acquiring sub-cellular/cellular resolution, location specific fatty acid and intact lipid data on a series of longitudinal sections of the planarian *Phagocata gracilis*. Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) imaging analysis of Z-sections taken at specific depths reveal the internal structures of these organisms based on chemical signals.

In ToF-SIMS, the surface chemistry is probed by bombarding a sample with primary ions which leads to ejection of secondary ions representing the chemical composition in the bombarded area. This primary ion beam can be rastered over the surface, acquiring individual mass spectra for each image pixel. ToF-SIMS is a label-free, semi quantitative analysis method that generates a chemical map of the surface providing information on localization and intensity of a large number of chemical species within one data set. Since ToF-SIMS analysis is an untargeted (label-free) approach, it can be used as discovery technique. A variety of primary ion beams is currently available, all with different properties concerning achievable spatial resolution in images as well as the intensity of high mass signals in the spectra [21]. As a rule of thumb, the higher the spatial resolution, the lower the mass of signals detected. This is due to the tendency of primary ion sources (e.g., Au⁺, Bi³⁺, Cs⁺) that are capable of submicron spatial resolution in images to significantly fragment molecules. Larger molecules, such as intact lipids or proteins typically need a larger analysis area per pixel to produce signals with sufficient intensities to allow for imaging [22]. In order to investigate the distribution of intact lipid species (masses of ~700–900 Da), a gas cluster ion beam (GCIB) capable of producing imageable secondary ion signals up to 2000 Da and spatial resolutions in images of a few μm was used [23,24]. GCIB ToF-SIMS has been used to study lipid distributions in a variety of samples such as mouse brains [25,26] and drosophila brains [27], human breast cancer [28] and infarcted mouse hearts [29]. In all cases, the combination of detailed spatial and chemical information provided new insights that no other method had previously provided. For example, in a

breast cancer study GCIB ToF-SIMS imaging results showed that lipid species PI(38:4) and fatty acid FA(20:4) were present mainly outside breast cancer growths, and are likely associated with inflammatory cells, instead of being part of the cancer lipid metabolism.

For planaria lipid data is sparse and no location specific data are available. Although planarians are known for their regenerative capabilities, the only study looking at lipids in regenerative processes [18] lacked spatial information and could only provide very general results for entire lipid classes. In this study, we thoroughly characterized all (detectable) lipid species present in planarians, studied their location in the organism, identified the organ where they are present and attempted to explain the significance of these lipids locations while putting previous data into context. These data are an important foundation for any future study focusing on lipid changes in planarians.

2. Materials and methods

Phagocata gracilis ($N = 3$) were analyzed with ToF-SIMS and reported in this study. Consecutive sections of specimen 1 were analyzed in positive and negative ion mode, specimens 2 and 3 were analyzed in negative ion mode. These data were supported with analyses of an additional 10 worms where either partial or full body scans were acquired in positive and negative ion mode. Fatty acid/lipid profiles were similar for all planarians and the results presented in this study are derived from all analyzed sections. Subsequent images of molecular distributions are shown mainly using specimen 1 (Fig. 1) because this specimen was cut in an orientation that aligns with traditional models of planarian morphology.

2.1. Sample preparation

Planarian flatworms, 5–8 mm in size were kept in aged water (stored at room temperature for several days to allow dissipation of chlorine) and starved one week before analysis to avoid interference from nonspecific signals derived from food products within the intestines. Due to the fragile nature of the worms and their deformation or active resistance when embedded into cryosection-media (optimum cutting temperature, Tissue-Tek® O.C.T. Compound, Sakura® Finetek), the following sample preparation protocol displayed in Fig. S1 was adopted: Prior to embedding, worms were sedated by being submerged into aged water containing 6% ethanol for 15 min to partially immobilize the planarians. As a mold for OCT embedding, a microscope slide with 4 detachable well chambers (MatTek Corp.) was used (Fig. S1b). Worms were placed onto the glass slide and laid down flat and straight using soft paint brushes. The worms were then surrounded by a sufficient volume of OCT to cover the sample while avoiding residual water to interfere with the sectioning and without providing the worm with a media to move around in and change its position, followed by rapid freezing in dry ice. Once frozen, the well chambers were filled with OCT and left on dry ice to solidify for 30 min. Well chambers with OCT blocks were detached from the microscope slide and solid blocks of OCT with the flat embedded worms were removed, exposing the ventral side of the worms. Worms were cut into longitudinal sections starting from the ventral (pharynx) to dorsal side (back) using a Leica CM1580 cryostat at -20°C with a section thickness of 10–15 μm . Consecutive sections were thaw mounted onto indium

tin oxide (ITO, Delta Technologies) coated glass slides and kept on dry ice until 1 h prior to analysis. On the day of analysis, samples were warmed up to room temperature under a gentle stream of nitrogen gas (industrial grade, Praxair, USA).

2.2. Microscopy

After slides were dry, a scan of the whole ITO slide was captured using an optical microscope (Invitrogen EVOS FL Auto Cell Imaging System) in combination with an in-house made sample holder to accommodate the ITO slides. Optical images were used to identify the tissue with the least amount of cracks and the presence of eye spots to indicate a central Z position within the worm where most organs are visible. These worm sections were used for the ToF-SIMS analysis.

2.3. Imaging mass spectrometry

ToF-SIMS imaging mass spectrometry analyses were performed using a J105 – 3D chemical imager (Ionoptika Ltd., UK). The J105 has been described in detail elsewhere [30,31]. Unlike conventional ToF-SIMS instruments, the J105 uses a quasi-continuous primary ion beam to produce secondary ions that are sampled by a linear buncher prior to ToF measurements. In the current study, a 40 keV gas cluster ion beam (GCIB) was used to bombard the sample with clusters of a gas mixture (15% CO₂ in Argon, Matheson, USA). The nominal selected cluster size was 4000 (Ar4000) although the gas used to produce the beam contained 15% CO₂ in all measurements.

Spectra were recorded over a mass range of 70–1000 Da with a mass resolution of 11,500 @ m/z 885.54 (FWHM). Large area images were generated by producing a series of individual image “tiles” that were stitched together automatically by the acquisition software. Each tile comprised 64×64 pixels and covered an area of $768 \times 768 \mu\text{m}^2$ for lower spatial resolution scans (12 μm per pixel, specimen 1 positive and specimen 3) and 256×256 pixels over the same area for detailed scans (3 μm per pixel, specimen 1 negative and specimen 2). In total, images were generated with a primary ion dose density of 5×10^{12} ions/cm² and in negative and positive ion mode on consecutive sections, covering areas of different sizes, depending on the varying body sizes of the worms. Detail images were generated using a primary ion dose of 1×10^{13} ions/cm² in negative ion mode. When the beam size was smaller than the pixel size, the shots were spread over each pixel to ensure an even primary ion dose density.

2.4. LC-MS/MS

LC analysis was performed as described by Kyle et al. [32]. Briefly, five worms were lysed separately by bead beating and via pressure cycling technology (PCT). Metabolite, protein, and lipid extraction (MPLEx) was performed, adapted from the method of Folch et al. [33] (Nakayasu et al.) [34]. The lipid extract was first separated using a reversed phase Waters CSH column and eluting molecules were analyzed in both positive and negative ionization in a Velos-ETD Orbitrap mass spectrometer (Thermo Scientific). The top four most intense ions per cycle were fragmented by collision-induced dissociation (CID40, 40% normalized collision energy). LC-MS/MS spectra were analyzed using the Thermo Excalibur Qual Browser software.

2.5. Imaging PCA

Multivariate analyses (principal components analysis, PCA) on ToF-SIMS images were performed on reduced data-sets in MATLAB (The MathWorks Inc.) using the NBToolbox (<https://www.nb.uw.edu/mvsa/nbtoolbox>). PCA is a multivariate analysis routine that defines a new coordinate system that captures the largest variance within a dataset [35,36]. PCA results are displayed as scores and loadings. The scores show the relationship between the samples (image pixels) and the loadings show which variables (peaks) are responsible for the differences seen within the scores. As PCA was mainly used to determine which areas of the worm showed unique distributions of various ions and was not the focus of this study, a more detailed description of PCA and the software used for this study is provided in the supplementary material.

3. Results and discussion

3.1. Overview of lipid head-group and fatty acid distribution in longitudinal planarian sections

Longitudinal sections of planarians were analyzed with imaging ToF-SIMS to map the lipidomic landscape in planarian worms. The data contained in the subsequent MS images were analyzed with PCA in order to identify chemically unique areas in scores images as well as the MS signals (peaks) associated with those areas in the loading plots. PCA results for the analysis of specimen 1 in positive and negative ion mode are shown in Fig. S2. PCA of the negative ion data shows a wide range of structural features with chemically unique signatures. PCA of the positive ion data shows some of the same structural features, but did not capture as many unique areas as the negative ion data. Comparing light microscopy images of the sections and MS/PCA scores-images to models, textbook drawings [11,12] and whole body in-situ hybridization staining [13], we were able to identify several organs: brain, intestines, pharynges (different to other planarian species, *Phagocata gracilis* has multiple small pharynges instead of one large central pharynx), testes, and several parts of the reproductive system. Signals with different distributions associated with specific organs were initially identified using PCA. These distributions were then confirmed by looking at the original MS-images. Studying the PCA loadings, we were able to identify unique lipid species present in each organ system, apart from the brain which seems to share all signals with the testes. In contrast, the testes show signals that are not present in the brain.

Visual examples detailing these distributions are shown in Fig. 1b–f where single ion images of lipid species uniquely present in the different organs are displayed along with an overlay of the spectra from these regions highlighting the lipid species shown in the images (Fig. 1g). A list of assignments for each detected molecular species, as well as relative intensity of these species in the different organ systems can be found in Table 1. Table S1 additionally contains lipid fragments, fatty acid locations and relative intensities. It was noted that most species occur in several organs but are most intense in one or two. These species are indicated by three stars in the tables. The selection criteria behind the mass assignments was based on mass (m/z of any given peak), mass resolution of $\sim 10,000$ and a mass accuracy of < 5 ppm. This however means chemical isomers as well as species with similar molecular weights cannot be distinguished. To gain confidence in the species assignment and avoid

assignments errors, the location of lower mass lipid related fragments as well as lipid head-groups was taken into consideration. MS images for the head-group specific fragments of phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylglycerol (PG) and phosphatidylinositol (PI) present in negative ion mode spectra as well as phosphatidylcholine (PC), sphingomyelin (SM) and other ceramide-species (Cer) in positive ion mode are shown in Fig. S3. Phosphatidylserine (PS) is not known to produce a unique head-group specific fragment and the identification of PS species is mainly based on neutral losses (M-87 Da). MS-images for all fatty acids can be found in Fig. S4. Additionally, where possible lipid identities were confirmed using LC-MS/MS and are indicated with (+) in Table 1. LC-MS/MS analysis of lipid species PI(18:0/22:4) is shown in Fig. S5. In some cases confirmation was not possible as there are no reference spectra or predicted fragments for those species available, however in no case did the LC-MS/MS spectra contradict the initial assignment.

In general, PI, PE, PC and PC/SM head-group fragments as well as a currently unidentified fragment at m/z 154.03 (proposed formula: $C_3H_9NO_4P^-$) are highly abundant in the tissue. Weak signals of PA and Cer/SM fragments are present. When studying the fatty acid profile and their distributions (Fig. S4) it is interesting to note that apart from those species usually detected in animal tissues (ester linked fatty acids with even number carbon atoms and 0–6 double bonds) planarians also contain fatty acids with an uneven number of carbon atoms (odd-numbered/branched fatty acids) as well as ether linked fatty acids or ether fatty acids. Lipids containing those fatty acids can be called ether lipids e.g. PC(O-16:0/18:1) or PS(P-16:0/16:0). There are two forms of ether linked lipids/FAs, plasmalogen- and plasmalogenyl-phospholipids. Plasmalogen lipids are ether linked in sn-1 position and labelled as e.g.: FA(O-18:0), FA(e18:0) or FA(a18:0). Plasmalogen lipids (trivial name: Plasmalogens) are ether linked next to a $C=C$ double bond in sn-1 position and are labelled as e.g.: FA(P-18:0) or FA(p18:0) [37]. Interestingly, it has been shown that plasmalogen (prefix O) linked fatty acids do not easily fragment and mainly produce [sn-1 + head-group] fragments in MS/MS studies rather than sn-1-fragments. Plasmalogen (prefix P) fragment like ester linked lipids [38]. Considering all analyses in this study were performed using gas cluster ions, which is a rather gentle form of SIMS, it is not surprising that no plasmalogen (O) linked fatty acids are found in the spectra but instead a number of higher mass plasmalogen (O) linked lipid fragments are seen (examples in Table S1). Compared to other organisms, planarians had a higher abundance of FA(22:5) (and FA(22:4)) which was also noted in previously published articles [17,19]. The odd-numbered fatty acids FA(15:0) and FA(17:0) are present in our sample (Table S1, Fig. S4). Previous studies had speculated that these FA species were due to contaminating bacteria in the planarian sample [17,19,20]. If bacteria were present, one would expect to find PG lipids as they are highly abundant in most bacteria [39]. However, there seems to be no m/z 209.03 (typical PG head-group fragment) in the sample. The unique distribution in the tissue as well as the absence of PG fragments suggests that these fatty acids are not associated with bacteria.

3.2. Lipids and fatty acids associated with different organs

3.2.1. Brain—The planarian central nervous system is located in close proximity to their eyespots (a region with dark pigmented sensory cells that are sensitive to light). The

eyespot can be easily identified in tissue sections allowing the brain to be readily located, even though it is not an obvious feature under the microscope without the aid of chemical stains. The main lipid head-group fragments found in the brain are PI and PC. Chemically, the brain can be distinguished from other tissues within the head by its intense PI(18:0/20:4) signal (m/z 885.55) as shown in Fig. 1b, as well as a species at m/z 871.57 (PI(O-18:0/20:4)). The lipid species PI(18:0/20:4) is highly abundant in vertebrate brains and the most common PI-species in mammalian cells [40,41]. PI(18:0/20:4) fulfills a number of important functions in cell membranes. This lipid contains arachidonic acid (FA(20:4)), a highly bent fatty acid chain that does not allow for tight packing of lipids, which helps maintain membrane fluidity and permeability and facilitates cell migration. Additionally PI(18:0/20:4) is preferentially converted to phosphatidylinositol polyphosphates (PIPs), which play key roles in a wide range of cellular processes, hence the occurrence in all organs. One example is 3,4,5-trisphosphate (*PIP3*) which when converted by *PI3* kinase triggers the activation of the *Akt* pathway, a mechanism most widely known for its involvement in cell proliferation and the promotion of cancer growth [42]. The molecular orthologue to *Akt* in planarians is the protein *Smed*. *Smed* has been found to play a pivotal role in neoblast migration and tissue maintenance in planarian regeneration [43].

3.2.2. Intestines—The planarian intestine is a highly branched structure reaching from the head region, along both sides of the pharynx to the end of the tail region. A number of unique signals precisely show this distribution within planarian tissue sections. For example, m/z 879.60 in negative ion mode (Fig. 1c) as well as a series of peaks separated by exactly 14.015 Da smaller/bigger than m/z 879.60 ($\pm$ CH₂) are shown in the purple spectrum in Fig. 1g and listed in Table 1. Database searches (e.g., Lipidmaps, HMDB, massbank) provide no identity for this cluster of peaks within 10 ppm accuracy while most other species are within 5 ppm. The spacing of 14 Da suggests that they are a group with the same molecular formula which contains fatty acid tails with different chain lengths. Another unique peak found in the intestines includes m/z 154.027. Co-localization of m/z 154.027 with the peak cluster around m/z 879.60 as well as its intensity suggests that m/z 154.027 is a common fragment of those peaks. Neither the higher mass species, nor the fragment at 154 Da, are reported in any publication or database, which suggests that they are a new class of not yet identified lipids. Using the molecular formula calculator ChemCalc (© Copyright 2014 EPFL - <http://cheminformatics.epfl.ch> - Luc Patiny) and limiting the input to the most common atoms found in organisms, the suggested molecular formula for the 154 Da compound is C₃H₉NO₄P. The fragments produced in LC-MS/MS studies also did not shed any light on the identity of these species.

In positive ion mode imaging PCA principal component 7/8 shows a ceramide fragment (m/z 264.27) enhanced in the intestines region, which is in agreement with previous studies finding that ceramide-synthase is required to produce functional phagocytes within planarian intestines [44].

3.2.3. Testes—The testes in planarians are numerous circular structures along both sides of the pharynx and can be visualized using staining methods [45]. The signals in ToF-SIMS images that show the strongest correlation with structures resembling the testes are m/z

911.57 PI(18:0/22:5) (Fig. 1e) and m/z 913.58 PI(18:0/22:4). The lipid assignment is based on the observation that these lipid peaks co-localize with the fatty acid species FA(22:4) and FA(22:5) and confirmed using LC-MS/MS. These fatty acids have been previously reported to be present in planarians in unusually high concentrations [17] and exhibit the highest intensity in the testes. Also uniquely localized to the testes are m/z 387.33 and m/z 385.32. The closest possible assignment that can be made for these masses are very long chain polyunsaturated fatty acids (VLCPUFA) FA(26:4) and FA(26:5). FA(24:X) or longer (with up to 6 double bonds) in testes has been observed in mammals via gas and liquid chromatography methods [46,47]. While most VLCPUFAs in mammals have been shown to be incorporated into sphingolipids, the 26 carbon chain length fatty acids are not incorporated into sphingolipids in a significant amount [48]. Our results are consistent with these previous studies as no elevated amounts of sphingolipid fragments (e.g., m/z 264.2 in pos. ion mode) were observed in the testes. The LC-MS/MS data shows species at m/z 387.33 and m/z 385.32 in the worm extracts but not as parts of lipid species, suggesting they could be free fatty acids. The FA(26:4/5) are elongation products of FA(22:4/5) using an enzyme called ELOVL2 (or its homologues). Beyond 26 carbon atoms, elongation happens via ELOVL4 [49]. Fatty acids with > 26 carbon atoms could not be detected in planarian sections. Therefore we can conclude that ELOVL4 is either not present or not active in the laboratory kept planarians.

3.2.4. Pharynxes—The distribution of pharynx lipids is shown in Fig. 2 for specimen 1 and 2. Although specimen 1 and 2 were analyzed largely under the same conditions, the fragile nature of the sample as well as day to day adjustments in instrument settings can lead to different image resolution in ToF-SIMS scans. In planarians, the pharynx (or pharynxes) is located centrally on the ventral side of the worm, reaching into the body center, while all other structures are organized around it. The pharynx has the three germinal cell layers as identified in microscopy images in Fig. 2b and d: ectoderm (ec: epidermis or pharynx cavity epithelial cells), mesoderm (in: inner muscles, pa: parenchyma and om: outer muscles.) and endoderm (en: gastrodermis or lumen epithelium) [50,51]. In ToF-SIMS, the three layers are found to have different chemistries.

The single layer of epithelial cells forming the ectoderm in the pharynxes and the outermost skin cells are dominated by a number of signals (m/z 718.50 [M-H]⁺ Fig. 2a and c red) which can all be assigned to ether lipid species PS(P-16:0/16:0) and its fragments. To exclude the possibility that these signals are associated with the mucus coating of planarians, a sample of the mucus was analyzed (data not shown). Only a small amount of lipid species associated with either the ectoderm or endoderm was found in this mucus as compared to the worm. Most ToF-SIMS signals stemming from the mesodermal structures of the pharynx can be found in at least one other organ as well as the connective and muscle tissue between the different organs as shown by m/z 885.55 PI (18:0/20:4) (Fig. 2a/c blue) which are all formed by the mesodermal cell layer. The most intense signal that is specific to the endoderm is another plasmalogen species PS(P-18:0/18:1) at m/z 772.55 (Fig. 2a/c green). The higher resolution ToF-SIMS scan from specimen 2 (Fig. 2c) shows that those signals are confined to the innermost lumen epithelium of the pharynx. A review by Nagan et al. [37] describes the biosynthesis of plasmalogen and what little is known about the functions of

different species in detail. Plasmalogens are synthesized in peroxisomes and are thought to increase membrane fluidity and facilitate membrane fusion. Little is known about the biological function of phosphatidylserine plasmalogens apart from being present in bacteria [52], macrophages [53] and the human retina as well as human ocular neurons where (among other lipid species) PS(P-18:0/18:1) and PS(P-16:0/18:1) have been detected and identified by Nagy et al. [54] using MS/MS. LC-MS/MS yielded the same fragment species. The species PI (O-18:0/18:1) at m/z 849.59 is partially co-localized with (P-16:0/16:0). This species is interesting, as its intensity increases towards the head region, while the PS(P-16:0/16:0) is confined in the pharynxes. PI (O-18:0/18:1) can also be found in part of the intestines that is located in the head region (and in the female reproductive organs). This suggests that the pharynx, used for ingestion, and the intestines, used for digestion, are connected close to the head.

In positive ion mode, all central organs (pharynx and reproductive system) show largely the same signals (highlighted in principal component 5 in Fig. S2 positive ion mode). In general, while all other structures produce intense signals in negative ion mode, the pharynx is dominated by PC species and produces intense signals in positive ion mode. Most notably is the presence of another ether lipid species, PC(O-16:0/18:1) at m/z 746.61 and its sodiated and potasiated version at m/z 768.59 and 784.57 respectively. These species have been previously identified in snail neurons and their identity was confirmed by Passarelli et al. using MS/MS [55].

3.2.5. Reproductive system—In ToF-SIMS, the distribution of lipids in the reproductive system shows a multitude of diverse signals, as shown in Fig. 1f. ToF-SIMS data for the reproductive system for specimen 1, 2 and 3 is shown in Fig. 3. Specimen 1 and 2 provide similar longitudinal views (Fig. 3a–f and m–n). Specimen 3 (Fig. 3g–l) was sectioned sagittal instead of longitudinal, providing a view of the copulatory apparatus, more closely resembling the original sketches of the *Phagocata gracilis* reproductive system by Kenk [56]. The planarian copulatory system is located in the center of the organism towards its posterior end and consists of a number of structures, best described and visualized by Collins et al. [57] and Stocchino et al. [58]. A detailed description including drawings of the reproductive system of *Phagocata gracilis* was generated by Peaslee (1910) [11]. Comparing the structures present in our sections (using light microscopy images Fig. 3a/g/m) to the above mentioned publications, we assigned the structures as seminal vesicles (sv), penis papilla (pp), penile bulb (pb), ejaculatory duct (ed) and common or female atrium (ca/fa). Those assignments are supported by comparing ToF-SIMS signals identified in specimen 1 with specimen 2 and 3 (Fig. 3i–l/n). Analysis of the posterior part of specimen 2 shows the whole penile bulb to be surrounded by a single layer of epithelial cells (Fig. 3n, m/z 239) which is in agreement with sketches of the planarian reproductive system as well as H&E stained sagittal sections of the reproductive system of closely related Platyhelminthes species [59]. This epithelial layer shows the same signals as the ectodermal epithelial layer around the pharynxes, so it is probably of ectodermal origin as well. The penile bulb is dominated by a number of intense PE-Ceramide signals (e.g. PE-Cer(d34:1) at m/z 659.51 Fig. 3d/k/n). This assignment is supported by the PE head-group fragment in LC-MS/MS measurements, the co-localization of the PE head-group fragment and a common ceramide

fragment with the reproductive system in ToF-SIMS images as shown in Fig. S3 as well as the absence of sphingomyelin (PC-Cer) fragments in positive ion mode spectra (PE-Cer and PC-Cer species have the same mass). The penile bulb is the muscle used to extend and retract the penis which also contains gland cells. The same PE-Cer signals can be found in the mesodermal layer of the pharynx, which contains several muscles as well as glandular cells in the parenchyma. Therefore we hypothesize that PE-Cer signals in planarians are not specific to the reproductive system, but rather an indicator for either muscular or glandular structures. Penis papilla, seminal vesicles and the common or female atrium (which is located posterior from the male reproductive organs) show intense signals for m/z 821.57 (PI(O-16:0/18:1), Fig. 3c/b) and m/z 849.59 (Fig. 3b/f/l/n) assigned to PI(O-18:0/18:1) and the ejaculatory duct produces a unique signal at m/z 677.53 (Fig. 3e/j) but also shares signals with the testes (m/z 911.56 as shown in Fig. 1e). RBGY images provide better visualization and show the localization of the lipid/associated fatty acid species to each other.

The two planarian ovaries are located close to the ventral side of the worm, roughly between the pharynx and the head region. In consecutive sections of specimen 1 starting from the ventral side to the center we found m/z 227.20 (FA14:0) and m/z 241.22 (FA15:0) predominantly localized ventrally at both sides of the pharynx (localization of FA(15:0) shown in consecutive sections in Fig. S6). Given the location of this signal we speculate that it is associated with the ovaries/oviducts. Due to a slight tilt during sectioning, they appear first on the right and then, as we cut further into the tissue, on the left side. Also, specimen 2 shows FA(14/15:0) highly localized in a ventral structure, co-localizing with m/z 805.57 and 821.57, signals found in the female atrium of the reproductive system. In central sections, FA(14/15:0) is more evenly distributed throughout the organism and it is present in the female atrium and less intense in the male reproductive structures. Another uneven chain fatty acid, FA(17:0) can be found in the planarian sections, mainly in ectodermal structures (Fig. S4 shows distributions for FA(15:0) and FA(17:0)). The presence of uneven or branched chain fatty acids has been previously detected in planarians, but the fatty acids were speculated to be due to a bacterial contamination of the sample [19]. Given the organ specific localization inside the planarian sections it is plausible that this is not a contamination but planarians do in fact contain those species.

3.3. Fatty acid comparison: planarians vs. food sources

It has been previously suggested that fatty acid content of planarians is limited to their dietary intake as they are not able to synthesize fatty acids de-novo [16]. Most laboratory kept planarian worms, including those in this study, are fed beef liver only. If planarians cannot synthesize fatty acids, then the fatty acid content in the planarians should, in theory, be similar to that of beef liver. To test this theory, different parts of regular store-bought beef liver were analyzed with ToF-SIMS using the same settings as for the worm analysis. Since the liver is heterogeneous, we analyzed different parts of the liver based on its appearance and consistency and labelled them as liver-meat, liver-fat and liver-blood. Overview spectra comparisons (m/z 100–1000) in positive and negative ion mode can be found in Fig. S7. Overall, meat and blood signals are very similar to each other in negative ion mode. In positive ion mode the blood spectra contains hemoglobin (Fig. S6e). The spectrum from the fatty parts of the liver is higher in saturated fatty acids and triacylglycerides (TAGs) (Fig.

S6f). A comparison between normalized food spectra and a planarian spectrum representing the entire tissue section and most organs shows little similarities between lipid spectra (m/z 700–1000) in positive and negative ion mode, as was expected. More interesting are the fatty acid differences between the food sources and the worm itself (Fig. 4a–d). Myristic acid (FA(14:0)), while found in the worm tissue, shows little signal in the beef liver (Fig. 4a). The lack of myristic acid in beef liver has been previously reported by Enser et al. [60]. This result suggests that, contrary to prior studies, free living planarians may have the ability to de-novo synthesize fatty acids, at least in some organs [16].

Plasmalogen fragment FA(P-16:0) and odd numbered species FA(15:0) are enhanced in planarians compared to their food source. The liver is known to contain the least amount of plasmalogens compared to other organs but typical for ruminants (cattle), does contain heptadecanoic acid (FA(17:0) at m/z 269.25 which appears in our liver spectra Fig. 4b) and traces of pentadecanoic acid (FA(15:0) at m/z 241.22) [37,61,62]. The origin of the fatty alcohol species, necessary to synthesize plasmalogens (both P- and O- species) is not fully understood. In mammals, it is speculated that fatty alcohol species are acquired via the diet, but for liver fed planarians, this is not an option. Fatty alcohols may be produced in peroxisomes (oddly enough, in mammals in the liver) by the enzyme FAR (fatty alcohol reductase) that preferentially reduces C16 and C18 fatty acyl-species [37,63]. The appearance of FA(15:0) and FA(17:0) in our laboratory kept planarians may be explained by their food source, although in ToF-SIMS spectra we cannot distinguish between odd numbered and branched fatty acids, since they have the same mass. Planarians in the wild feed on decaying organisms and bacteria that are high in branched fatty acids. This may explain the presence of FA(15/17:0) in those worms [17,19] but the specific location of those species in the organism suggests a biological function. In *C. elegans*, branched fatty acids are important for growth and development [64]. Branched fatty acids are catabolized in peroxisomes. Little information is available about peroxisome activity in planarians, but a peroxisome targeting enzyme is active in ovaries (where we found FA(15/17:0)) during sexual maturation [65].

Most animals do not possess the enzymes necessary to produce linoleic (FA(18:2n6)) and α -linolenic acid (FA(18:3n3)) which are needed to synthesize all other omega-6 and 3 fatty acids in the body. Instead, most animals rely on plant sources for these fatty acids. Usually the lack of those essential fatty acids leads to an increase of mead acid (FA(20:3n9) [66]. *C. elegans* has been shown to be able to synthesize arachidonic acid FA(20:4n6) and eicosapentaenoic acid FA(20:5n3) on their own but are unable to elongate fatty acids beyond 20 carbon atoms [67]. The increased quantities of FA(18:3) (Fig. 4b), FA(20:4), FA(22:4) and FA(22:5) (all downstream products of linoleic acid, Fig. 4c) suggest that planarians are able to produce omega-6 fatty acids but the lack of FA(20:5) and FA(22:6) (downstream products of α -linolenic acid) indicates that they do not have an omega-3-desaturase. Planarians caught in the wild which had access to a variety of food sources show high amounts of FA(18:4) FA(20:5) and FA(22:6) [19,20], indicating that elongases as well as delta 4, 5, and 6 desaturases are highly active and will modify the C18 species available, either linoleic and α -linolenic acid, or just linoleic acid. A review by Lee et al [68] describes the current knowledge PUFA synthesis in detail. FA(26:4), another downstream product of

linoleic acid, not present in the liver spectra (Fig. 4d), is found in planarian testes. We speculate that in wild planarians FA(26:6) and FA(26:5) would be more prevalent.

3.4. Planarian genetics

The planarian genome is notoriously difficult to sequence as standard DNA isolation procedures perform poorly on planarians and the genome is A/T rich and highly repetitive (61.7% repetitive fraction) [69]. This is probably the reason why there are limited planarian data in databases like Uniprot and no data in KEGG as well as no information on fatty acid/lipid associated genes or proteins. One recent study where the genome of the planarian *Schmidtea mediterranea* was sequenced, found that planarian genomes are very different from any other genome previously sequenced and similarities are limited to certain domains in proteins. They also state that while planarians likely have > 1000 planarian-specific protein coding genes they lack a great number (452) of “essential” genes. For example, they were found to lack key components for DNA double-stranded break (DSB) repair [69] even though they have an extraordinary resistance to DSB-inducing γ -irradiation [70]. Similarly planarians seem to lack fatty acid synthesis genes such as FASN.

PlanMine [69] even though they have an extraordinary resistance to DSB-inducing γ -irradiation [71] is a database containing the *Schmidtea mediterranea* genome as well as transcriptomes from several other planarian species. PlanMine also has the option to BLAST (Basic Local Alignment Search Tool) search against these data for gene/protein homologues. We compared *C. elegans* proteins involved with fatty acid/lipid synthesis, as described by Zhang et al. [69] even though they have an extraordinary resistance to DSB-inducing γ -irradiation [72], to the planarian gene/protein data in PlanMine and found that no homologue for *fasn-1* is present in the planarian. However, a BLAST search (using PlanMine standard settings) produces a highly significant hit for *pod-2* and shows a protein containing an Acetyl-CoA carboxylase domain. *Pod-2* is predicted to catalyze the first step in de novo fatty acid biosynthesis [72, 73] in *C. elegans*. This could explain the presence of myristic acid (FA(14:0)) in planarians even though it is not found in their beef liver diet. Other homologues present are *ads-1* and *lis-1*, which are involved in ether lipid metabolism and potentially a homologue to human FAR, the fatty acid reductase enzyme that preferentially reduces C16 and C18 fatty acyl-species [37,63] which are ether fatty acid precursors. The presence of *ads-1* and *lis-1* could explain the high amount of unusual ether lipid species in the sample (e.g. PS(P-16:0/16:0 in the pharynx). *Elo* genes, involved with elongation and production of branched fatty acids are not found in the planarian genome and scores for proteins involved in converting FA(20:4) to FA(20:5) are ambiguous, as are searches for ELOVL2 and ELOVL4, the enzymes responsible for prolonging fatty acids beyond 24 and 26 carbon atoms. A comparison of the ToF-SIMS data of lipids in the planarian food to the planarian indicates that elongation and desaturation of fatty acids must happen in planarians. However, the genes involved are not homologues to the ones found in *C. elegans*. These examples show that planarians must be able to create fatty acid types, even though they lack the analogous genes that are common in other organisms (such as *fasn-1*).

4. Conclusion

Information on lipids in planarians is sparse and no location specific data were previous available. Location specific chemical information is crucial in order to elucidate the significance and function of lipid species in organisms and tissues. Different to targeted approaches, ToF-SIMS is a label-free technique which enables us to use it as discovery method. Here we demonstrate the capability of ToF-SIMS in combination with high energy gas cluster ion beams to image intact lipid species in planarian tissue sections. The untargeted, subcellular-location specific chemical information presented here enables us to study all detectable lipids in the sample, even those stemming from a single layer of cells like in the pharynx. We can study the distribution of common (PI, PC), “unusual” (PS-ether lipids) and even completely unknown species of lipids within one analysis, which wouldn't be possible in a targeted experiment. Discoveries of similarities between planarians and other species, such as the presence of VLCPUFA in both planarian and mammalian testicles would not have been possible without the spatial context for the chemical data. Additionally, these data elucidates differences between planarians and mammals, such as the higher amounts LCFAs in planarians as well as the presence of the yet to be identified, potential lipid species in planarian intestines. A comparison of the fatty acid species in planarians and its only food source, beef liver, and the presence of certain fatty acid metabolism genes like pod-2 in the planarian genome, challenges previous findings as it provides us with evidence that planarians are capable of de-novo fatty acid synthesis. This publication demonstrates the power of ToF-SIMS imaging for biological analysis. The data presented here gathered from wild type planarians living in ideal conditions, can be used as a basis to elucidate the function of different organs in planarians as well for future studies on the involvement of specific lipid species in various processes such as regeneration, responses to toxins and environmental changes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Localization of different lipid species associated with planarian organs. (a) Light microscope image of longitudinal planarian tissue section (specimen 1) on ITO-glass, (b–f) false color lipid distribution images generated using ToF-SIMS analysis of this tissue section, brighter color equals higher intensity. (b) Distribution of PI(18:0/20:4) at m/z 885.55 highlighting brain and reproductive organs, (c) Unidentified species at m/z 879.60 uniquely present in the intestines, (d) PS(P-16:0/16:0) at m/z 718.51 present in ectodermal epithelial cells in skin and pharynx as well as parts of the reproductive system, (e) PI(18:0/22:5) at m/z 911.56 mainly present in the testes, (f) PE-Cer(d34:1) at m/z 659.51 (red, RO1 in Table 1) highlighting the penile bulb, unidentified species at m/z 947.69 (orange, RO2 in Table 1) in the penis papilla and seminal vesicle and PI(O-18:0/18:1) at m/z 849.59 (yellow) most intense in the female atrium and skin. (g) Overlay of negative ion mode Σ ToF-SIMS spectra extracted from pixels representing each of the areas shown in b–f, normalized to the size of the extraction area, with the same color scheme as in the images, the peaks displayed in the images are highlighted.

Fig. 2.

Planarian pharyngeal structures in microscope and MS-images. (a) RGB overlay ToF-SIMS image of specimen 1, red: PS(P-16:0/16:0) at m/z 718.51, green: PS (P-18:0/18:1) at m/z 772.55, blue: PI(18:0/20:4) at m/z 885.55. The white square shows the part of the image displayed in b. (b) Microscopy image of specimen 1, endodermal (en) and ectodermal (ec) structures in skin and pharynx indicated with arrows. (c) RGB overlay ToF-SIMS image of specimen 2, showing the same species as in A. The white square shows the part of the image displayed in d. (d) Microscopy image of specimen 2, ectoderm (ec), outer muscle (om), inner muscle (im) and endoderm (en) of the pharynx in skin and pharynx indicated with arrows, parenchyma (pa) labelled.

Fig. 3.

Microscope and MS images highlighting structures of the planarian reproductive system. (a) Microscope image of specimen 1 with the penis papilla (pp) outlined, seminal vesicles (sv), ejaculatory duct (ed), penile bulb (pb) and testes (tt) and central or female atrium (ca/fa) indicated with arrows. (b) RGBY overlay ToF-SIMS image of Specimen 1 (m/z values stated in the image). (c–f) Specimen 1 single ion images of peaks shown in the right upper corner with the structure where those species are most intense listed in the lower left corner. (g) Microscope image of sagittal section specimen 3. (h) RGBY overlay ToF-SIMS image of Specimen 3 (m/z values stated in the image). (i–l) Single ion images of specimen 3 (peaks shown in the right upper corner). (m) Microscope image of longitudinal section specimen 2, area shown in ToF-SIMS image n indicated with dashed lines. (n) RGBY overlay ToF-SIMS image of specimen 2 (masses stated in the image). Mass assignments can be found in Tables 1 and S1. Note: color assignments in RGBY overlay images are not consistent between images but were chosen from a list of colocalizing species for clarity and to ideally represent each structure.

Fig. 4.

Overlay of ToF-SIMS spectra from food sources vs. planaria. ToF-SIMS spectra acquired in negative ion mode, normalized to total signal, red: summed signal of a whole planarian tissue section (worm), green/black/grey: various parts of beef liver. Certain fatty acid species labelled with the marker color indicating which spectra has the highest relative intensity, a) m/z 225–245, b) 260–280, c) 300–340, d) 385–395. Complete spectra are shown in Fig. S7.

Table 1

Lipid species present in different planarian organs.

NEG	Brain	Int.	Phar.	Test.	RO1	RO2	Assignment	Formula	Exact	ppm	LC
947.692	*					**					
913.577				***	*		PI(18:0/22:4) [M-H] ⁻	C ₄₀ H ₈₆ O ₁₃ P	913.58	-3.47	+
911.567				***	*		PI(18:0/22:5) [M-H] ⁻	C ₄₀ H ₈₄ O ₁₃ P	911.56	1.95	+
885.549	***		*	***	**		PI(18:0/20:4) [M-H] ⁻	C ₄₇ H ₈₂ O ₁₃ P	885.55	-0.76	+
883.532	**			**	**		PI(18:0/20:5) [M-H] ⁻	C ₄₇ H ₈₀ O ₁₃ P	883.53	-1.91	+
879.608		***					Unidentified, co-localizing with 891.6332, 893.6245, 905.6551, 907.6402, 919.6719, 921.6548, 931.6660, 933.6831, 935.6598, 945.6873, 949.6857				
877.618		**	*		*	*	PI(37:1) [M-H] ⁻	C ₄₇ H ₉₀ O ₁₂ P	877.62	0.96	+
871.57	***			***	**		PI(O-18:0/20:4) [M-H] ⁻	C ₄₇ H ₈₄ O ₁₂ P	871.57	0.28	+
863.567		**	**	***	***		PI(18:0/18:1) [M-H] ⁻	C ₄₅ H ₈₄ O ₁₃ P	863.56	2.17	+
861.548		***			***		PI(18:0/18:2) [M-H] ⁻	C ₄₅ H ₈₂ O ₁₃ P	861.55	-1.36	+
857.521	*		*	***	*	**	PI(16:0/20:4) [M-H] ⁻	C ₄₅ H ₇₈ O ₁₃ P	857.52	3.68	+
851.584		***				***					
849.586			*	**		***	PI(O-18:0/18:1) [M-H] ⁻	C ₄₅ H ₈₆ O ₁₂ P	849.59	0.87	+
838.559	*	*		*	*	*	PS(18:0/22:4) [M-H] ⁻	C ₄₆ H ₈₁ NO ₁₀ P	838.56	-1.05	+
835.578		***				***	PI(34:1) [M-H] ⁻	C ₄₄ H ₈₄ O ₁₂ P	835.57	9.2	
821.562						***	PI(O-16:0/18:1) [M-H] ⁻	C ₄₃ H ₈₂ O ₁₂ P	821.55	9.3	+
812.536		**		*		**	PS(38:3) [M-H] ⁻	C ₄₄ H ₇₉ NO ₁₀ P	812.54	-10.4	+
810.531		**	*	*	*	*	PS(18:0/20:4) [M-H] ⁻	C ₄₄ H ₇₇ NO ₁₀ P	810.53	3.11	+
794.562	*	**		*	*	*	PE(18:0/22:4) [M-H] ⁻	C ₄₆ H ₈₁ NO ₈ P	794.57	-9.75	+
792.554	**	*	*	***	**	*	PE(18:0/20:5) [M-H] ⁻	C ₄₅ H ₇₉ NO ₈ P	792.55	-1.01	+
788.542	*	**		**	**	*	PS(18:0/18:1) [M-H] ⁻	C ₄₂ H ₇₉ NO ₁₀ P	788.54	-2.57	+
786.528	*	**		**	**	*	PS(36:2) [M-H] ⁻	C ₄₂ H ₇₇ NO ₁₀ P	786.53	-0.1	+
778.574	***	**	*	***	**	***	PE(P-18:0/22:4) [M-H] ⁻	C ₄₃ H ₈₁ NO ₇ P	778.58	-1.84	+
772.553			**				PS(P-18:0/18:1) [M-H] ⁻	C ₄₂ H ₇₉ NO ₉ P	772.55	4.19	
766.54	*	*		**	**		PE(38:4) [M-H] ⁻	C ₄₃ H ₇₇ NO ₈ P	766.54	2.15	+
750.539	*	*		**	**	**	PE(P-16:0/22:4) [M-H] ⁻	C ₄₃ H ₇₇ NO ₇ P	750.54	-6.58	+

NEG	Brain	Int.	Phar.	Test.	RO1	RO2	Assignment	Formula	Exact	ppm	LC
748.529	**	*		***	***	**	PE(P-16:0/22:5) [M-H] ⁻	C ₄₃ H ₇₅ NO ₇ P	748.53	0.55	+
743.603		*	*	***	***		PE-Cer(d40:1) [M-H] ⁻	C ₄₂ H ₈₄ N ₂ O ₆ P	743.61	-4.93	
729.585		*		***	***		PE-Cer(d39:1) [M-H] ⁻	C ₄₁ H ₈₂ N ₂ O ₆ P	729.59	-8.52	
718.503			***				PS(P-16:0/16:0) [M-H] ⁻	C ₃₈ H ₇₃ NO ₆ P	718.5	1.48	+
715.574		*	*	***	***		PE-Cer(d38:1) [M-H] ⁻	C ₄₀ H ₈₀ N ₂ O ₆ P	715.58	-1.8	
700.525			**	***	***		PE(P-16:0/18:1) [M-H] ⁻	C ₃₉ H ₇₅ NO ₇ P	700.53	-4.69	+
687.542			*	*	***	*	PE-Cer(d36:1) [M-H] ⁻	C ₃₈ H ₇₆ N ₂ O ₆ P	687.54	-2.87	+
673.528			*	*	***	*	PE-Cer(d35:1) [M-H] ⁻	C ₃₇ H ₇₄ N ₂ O ₆ P	673.53	-1.22	+
659.514			*	*	***	*	PE-Cer(d34:1) [M-H] ⁻	C ₃₆ H ₇₂ N ₂ O ₆ P	659.51	1.86	+
387.328			***				FA(26:4)	C ₂₆ H ₄₃ O ₂	387.33	3.39	
223.001	**	*	*	***	**	*	PI-Fragment	C ₆ H ₉ O ₇ P	223	1.05	
196.038	**	*	*	**	**	*	PE-Fragment	C ₅ H ₁₁ O ₅ NP	196.04	0.09	
171.006	*	*		*	*		Glycerol-3-phosphate	C ₃ H ₈ O ₆ P	171.01	1.23	
154.027		***					Fragment	C ₃ H ₉ NO ₄ P	154.03	-2.72	
140.012	*		*	*	***	*	PE Head-group	C ₂ H ₇ PNO ₄	140.01	2.79	
POS	Brain	Int.	Phar.	Test.	RO1	RO2	Assignment	Formula	Exact	ppm	LC
858.523					*	*	PS(18:1/22:5) [M + Na] ⁺	C ₄₆ H ₇₈ NO ₁₀ PNa	858.53	-3.72	+
848.559		*			*		PC(38:4) [M + K] ⁺	C ₄₆ H ₈₄ NO ₈ PK	848.56	1.97	+
846.541			**				PC(38:5) [M + K] ⁺	C ₄₆ H ₈₂ NO ₈ PK	846.54	-0.69	+
834.592			*		*	*	PC(38:3) [M + Na] ⁺	C ₄₆ H ₈₆ NO ₈ PNa	834.6	-8.68	
832.578	*	*	*	*	*	*	PC(38:4) [M + Na] ⁺	C ₄₆ H ₈₄ NO ₈ PNa	832.58	-5.76	
812.599			**		*		PC(O-18:0/18:1) [M + K] ⁺	C ₄₄ H ₈₈ NO ₇ PK	812.59	6.63	+
810.598	*	*	*	*	*		PC(18:0/18:1) [M + Na] ⁺	C ₄₄ H ₈₆ NO ₈ PNa	810.6	-0.67	+
808.584			**			**	PC(38:5) [M + H] ⁺	C ₄₆ H ₈₃ NO ₈ P	808.59	-1.73	
796.618	*		**		**	*	PC(O-18:0/18:1) [M + Na] ⁺	C ₄₄ H ₈₈ NO ₇ PNa	796.62	-1.73	+
788.624	*	*		*	*		PC(18:0/18:1) [M + H] ⁺	C ₄₄ H ₈₇ NO ₈ P	788.62	8.63	+
784.567	*		***		***	**	PC(O-16:0/18:1) [M + K] ⁺	C ₄₂ H ₈₄ NO ₇ PK	784.56	5.84	+
782.568	*	*	*		*		PC(34:1) [M + Na] ⁺	C ₄₃ H ₈₂ NO ₈ PNa	782.57	1.09	
774.634	**	*	**		**		PC(O-18:0/18:1) [M + H] ⁺	C ₄₄ H ₈₈ NO ₇ PH	774.64	-4.82	+

NEG	Brain	Int.	Phar.	Test.	RO1	RO2	Assignment	Formula	Exact	ppm	LC
768.585	*		***		**	*	PC(O-16:0/18:1) [M + Na] ⁺	C ₄₂ H ₈₄ NO ₇ PNa	768.59	-3.75	+
760.615			*		**	*	PC(O-17:0/18:1) [M + H] ⁺	C ₄₂ H ₈₂ NO ₈ PH	760.62	-9.15	+
746.605	**		**		***	**	PC(O-16:0/18:1) [M + H] ⁺	C ₄₂ H ₈₅ NO ₇ P	746.61	-1.79	+
369.353	**	**		**	**	**	Chol. [M + H-H ₂ O] ⁺	C ₂₇ H ₄₅	369.35	3.15	
264.27	*	*			*		Cer/SM Frag. C18	C ₁₈ H ₃₄ N	264.27	4.39	
184.074	***	*	***	**	***	**	PC-Fragment	C ₅ H ₁₅ NPO ₄	184.07	-0.03	

Listed are: the detected masses in ToF-SIMS spectra, negative and positive ion mode; the relative intensity in different organs, brain, intestines, pharynx, testes and 2 areas in the reproductive organs (RO1/2) indicated with *** (high), ** (medium), * (low) or blank for NO_x present; the assignment and detected ion species; the formula of this species; exact mass; and ppm difference between detected and exact mass. (+) in the LC column indicates that this species was confirmed using LC-MS/MS; -d- and -l- refer to 1,3-dihydroxy and 1,3,4-trihydroxy long-chain bases in sphingolipids, respectively.